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14. ABSTRACT
In this project, the VaxCelerate Consortium completed the generation and testing of a new vaccine against Lassa fever using its self-assembled vaccine platform. This process, starting with the provision of the genome for the Josiah strain of Lassa by DARPA and ending with the reporting of a validated immunogenicity end point from a transgenic HLA DR3 mouse vaccination model, was completed in 123 days and met its primary immune end point of showing significantly increased CD4+ T cell interferon gamma secretion in response to Lassa peptides. This "live fire" vaccine test demonstrated the capability of this distributed vaccine development consortium to rapidly

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Report Title

Rapid Generation and Testing of a Lassa Fever Vaccine Using VaxCelerate Platform

ABSTRACT

In this project, the VaxCelerate Consortium completed the generation and testing of a new vaccine against Lassa fever using its self-assembled vaccine platform. This process, starting with the provision of the genome for the Josiah strain of Lassa by DARPA and ending with the reporting of a validated immunogenicity end point from a transgenic HLA DR3 mouse vaccination model, was completed in 123 days and met its primary immune end point of showing significantly increased CD4+ T cell interferon gamma secretion in response to Lassa peptides. This “live fire” vaccine test demonstrated the capability of this distributed vaccine development consortium to rapidly produce and test a novel vaccine of relevance to public health responses. In parallel with this effort, the consortium produced and tested a modified version of its self-assembling vaccine protein that used a subunit of the full Mycobacterium tuberculosis heat shock protein 70. This assessment showed that the subunit was soluble but did not show the same potential for inducing DC maturation and cross presentation as the full length protein. This avenue of exploration was therefore concluded.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

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Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

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TOTAL:

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Received Book

TOTAL:

Received	Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Mark C Poznansky	0.15	
FTE Equivalent:	**0.15**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period:	0.00
The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:.....	0.00
Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):.....	0.00
Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:.....	0.00
The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense	0.00
The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:	0.00

Names of Personnel receiving masters degrees

NAME
Total Number:

Names of personnel receiving PHDs

NAME
Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED
Timothy Brauns	0.29
Pierre LeBlanc	0.35
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1 b. Office of Sponsored Programs
4333 Brooklyn Avenue NE
Seattle WA 981959472

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Patent Clause Number (d-1):
Patent Date (d-2):
Work Description (e): In silico analysis of Mtb HSP70 subdomains for modification
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Sub Contract Est Completion Date(f-2):

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1 a. University of Washington

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Seattle WA 981054696

Sub Contractor Numbers (c): 222357
Patent Clause Number (d-1):
Patent Date (d-2):
Work Description (e): In silico analysis of Mtb HSP70 subdomains for modification
Sub Contract Award Date (f-1):
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Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

Grant Title:	Rapid Generation and Testing of a Lassa Fever Vaccine Using VaxCelerate Platform
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Abbreviations and Acronyms

EID: Emerging infectious disease

GLP: Good laboratory practices

GMP: Good manufacturing practices

GxP: Good practices

HLA: Human leukocyte antigen

MAV: *Mycobacterium tuberculosis* heat shock protein 70-avidin fusion protein

MAVD: *Mycobacterium tuberculosis* heat shock protein 70 ATPase domain-avidin fusion protein

MGH: Massachusetts General Hospital

MtbHSP70: *Mycobacterium tuberculosis* heat shock protein 70

SAV: Self-assembling vaccine

List of Illustrations and Tables

I. Statement of Problem Studied

A. Challenge

The role of vaccines as a significant part of the Federal government's treatment response to the threat of abruptly appearing emerging infectious diseases (EIDs) with epidemic or pandemic potential has been limited. In response to two recent failures to deploy vaccines until an epidemic threat had largely passed—SARS in 1993 and H1N1 in 2009—the government facilitated the development of rapid vaccine production capabilities. While this investment has significantly increased the nation's capacity to respond to a range of pandemic influenza threats (see for example BARDA 2011), our nation's capabilities to rapidly deploy vaccines in the face of rapidly-appearing pathogen targets beyond influenza is open to debate. The predominant focus of current EID countermeasure development has been on the development of broadly-applicable post-exposure therapeutics (Hayden 2011).

The ability to rapidly deploy vaccines against an unanticipated pathogen in a relatively short period of time is challenging for a number of reasons. First, the diversity of pathogens (viral, bacterial, fungal, parasitic) and the corresponding range of human immune response makes it difficult to create a "one size fits all" vaccine approach. A recently published study examining potential immunologic markers of vaccine protection concluded that "delineating the molecular mechanism underlying immunity induced by different types of vaccines poses a great challenge" (Li *et al.* 2013). This diversity of pathogenic targets and host immune response often results in significantly different vaccination approaches applied to each type of pathogen, resulting in vaccines that are quite structurally diverse. The resultant proliferation of vaccine structures complicates vaccine formulation and manufacturing requirements and results in new regulatory questions being raised for each new candidate. Such complexity in vaccine development significantly diminishes the prospects that a vaccine approach could be reasonably used to address the challenge of abruptly appearing EIDs.

B. Approach

The current project was conceived as a means of addressing this vaccine development challenge. It sought to build a vaccine platform with the following characteristics that would address the challenges outlined above:

1) Applicable to a wide range of pathogen types.

2) Consistency of vaccine architecture across different specific vaccine candidates.

3) Capable of rapid, scalable production within a short period of time.

4) Does not require addition of adjuvants.

There is a significant challenge in developing a vaccine that is made essentially the same way each time but is capable to producing effective vaccine responses to a range of pathogens, and to do this without the use of additional adjuvants. This challenge is compounded by the need for rapid scalable production. The platform conceived to simultaneously address these requirements consisted of the following:

- A self-assembling protein that does not vary from vaccine to vaccine, but is capable of presenting antigens to both class I and class II antigen presentation pathways and provides immunostimulation without the use of additional adjuvants

- A pathogen-targeting set of peptides that can be changed from vaccine to vaccine in the embodiment of its specific class I and class II epitopes, but is structured the same for each vaccine.

- An integrated, *in silico* tool set for prediction of class I and class II epitopes that are immunogenic for humans, highly conserved across the pathogen, and not likely to induce cross reactivity with human self-epitopes or epitopes of commensal flora.

- Human-informed vaccine assessment tools for predicting and assessing immunogenicity and reactogenicity in humans, which include *in vitro* HLA binding assays, *in vivo* transgenic HLA mouse models, and *in vitro* human immunogenicity assays.

The first DARPA-funded project (VaxCelerate I) provided the opportunity to bring these elements together for the first time. This project provided the team practical experience with the separate technologies and identified technical aspects of the platform that required improvement to yield an effective vaccine. Specific changes in platform design that were discussed in the final report for VaxCelerate I were assessed and implemented for VaxCelerate II. The current project (VaxCelerate II) continued to emphasize the use of vaccine designs optimized for human response and assessment tools capable of providing early evidence of human effectiveness and safety. In addition, in VaxCelerate II we continued to practice a quality philosophy emphasizing progressive implemention of GLP/GMP standards for component supply, construction of the final vaccine and the testing of the vaccine, so that data generated would be reliable, reproducible and appropriate for submission as part of an IND application.

C. Project Goals and Tasks

Building on the initial effort in VaxCelerate I, this project (VaxCelerate II) mainly involved the demonstration that the VaxCelerate self-assembling platform could be used to build a new vaccine “from scratch”, starting with only the pathogen genome, and that this vaccine could be rapidly generated and shown to yield significant immunogenicity in a human-relevant animal model. This process would be conducted under elevated levels of quality to increase the level of confidence in the study result. In parallel with this main goal, the project also included conducting initial testing of modified versions of the self-assembling protein used in the SAV that might offer similar functionality while reducing the potential for unwanted responses.

The project included two specific Tasks: 1) 120-day Lassa fever vaccine generation and testing demonstration; and 2) development of VaxCelerate platform capabilities.

D. Project Team

This project was accomplished through the collaboration of six consortium members. Each provided a key element for the development or testing of the Lassa vaccine or the development of second-generation vaccine proteins. Table 1 summarizes these organizations, their roles and the key technologies provided as part of the project.

The team was integrated for the two tasks under the direction of the Vaccine and Immunotherapy Center at Massachusetts General Hospital. The projecct was organized in the following way: EpiVax, 21st Century, Pfenex, MGH and MPI collaborated on performing the initial vaccine generation and testing, while MGH and University of Washington worked on design of new vaccine self-assembling proteins in parallel. Coordination of the project was provided by a steering committee composed of representatives from 21st Century, EpiVax, MGH, Pfenex and U. Washington that met through teleconferences on a fortnightly basis; MGH provided project oversight and operational management.

Table 1: Project Organizations, Roles and Key Technologies

Organization Name (Location)	Project Role	Key Technology
Massachusetts General Hospital (Boston, MA)	Project management Self-assembly and characterization of vaccine Generation of pilot batches of HSP70 subunit-based self-assembling vaccine protein	Self-assembling vaccine platform produced under early-phase GMP
EpiVax, Inc. (Providence, RI)	Provide targeting epitope peptides for vaccine Assessment of immune response to vaccine (transgenic HLA model)	*In silico* vaccine epitope identification and optimization tool kit HLA transgenic mice
Pfēnex, Inc. (San Diego, CA)	Express and purify self-assembling protein moiety	Protein expression and purification under early phase GMP
21st Century Biochemicals (Marlborough, MA)	Generate 6 peptides for vaccine	Peptide synthesis and purification under early phase GMP
MPI Research (Mattawan, MI)	Mouse vaccine study and flow cytometry immunology assays	Animal studies and immunologic assays under cGLP
University of Washington (Seattle, WA)	*In silico* modeling of the structural integrity of HSP70 subunit proteins	*In silico* protein design tools

E. Deliverables and Timelines

The project had six deliverables:

1. Formulated Lassa fever vaccine with batch record and final reports on result of mouse vaccination study and ex vivo immunization study.
2. Complete amino acid sequences for all peptides.
3. One recombinant self-assembling construct protein and related batch record.
4. Individual peptide complexes and related batch records.
5. Reports on responses to the vaccination study.
6. The gene sequence of two modified self-assembling construct proteins and a report of *in vitro* characteristics.

These were delivered in the course of the project. This report focuses on the three most important results—rapid completion of a vaccine candidate, immunogenicity of the vaccine candidate, and analysis of the initial subunity protein for the self-assembling construct.

II. Summary of Important Results

A. 120-day Lassa fever vaccine generation and testing demonstration

Starting with the receipt of the genome of a Lassa vaccine strain, the team generated a self-assembled Lassa fever vaccine containing MHC class I and class II peptides derived from an *in silico* predictive modeling process and completed testing of the vaccine in a transgenic HLA DR3 mouse model to evaluate the ability of the vaccine to induce significant Th1 $CD4^+$ T cell response against the constituent Lassa peptides. This process was to be completed in 120 days.

The approach to this Task was to integrate the services and technologies of five different collaborators in a tightly-managed vaccine development and testing process. For this Task, EpiVax applied it's *in silico* predictive tools to identify a series of class I and class II peptides for Lassa fever. After selection of the final concatenated peptides by the VaxCelerate team, 21st Century produced the six full peptides for the vaccine using a standard solid-state synthetic process and purified the final peptides to above 95% purity. Production was accomplished without using trifluoracetic acid and was done under early-phase GMP standards. Each peptide was shipped with a specifications document that included a graph of the mass spec analysis of the final peptide.

In parallel with this process, Pfēnex produced the self-assembling MAV protein to be used in the vaccine. Its manufacturing was conducted under early phase GMP standards and included a complete batch record for the process. A total of 10 mg of the MAV protein was delivered with a purity above 95% and with an endotoxin level below the FDA standard for recombinant proteins.

MGH assembled, purified and formulated the final Lassa vaccine under early-phase GMP conditions. Vaccine characterization studies were performed under early-phase GLP. The endotoxin level of the final vaccine was below FDA standards for protein vaccines. A complete batch record for the vaccine was produced.

MPI Research performed the vaccination study using transgenic HLA DR3 mice provided through an agreement with EpiVax. For this study, MPI developed a cGLP-validated flow cytometry assay for determining interferon gamma expression by T cells and performed the mouse study under cGLP. MPI sacrificed the animals at the end of the study, harvested spleens and lymph nodes, isolated immune cells, stimulated these cells with peptide or control, and conducted the flow cytometry studies. MGH performed statistical analysis of these data generated by this study. Figure 1 shows the flow diagram for this Task.

1. Peptide Identification

The Lassa vaccine task started with the notification from DARPA that we were to use the genome for the Josiah strain of Lassa. Starting with this information, EpiVax completed a process to identify potential class I and class II epitopes for use in the vaccine in an eight-day period. Here is the process they employed in brief.

Step 1: EpiMatrix Analysis. EpiVax downloaded the amino acid sequence of the input Lassa Josiah genome from GenBank and loaded the relevant protein sequences into the online iVAX vaccine design tool. All input proteins were parsed into 9-mer, overlapping frames and analyzed using the EpiMatrix algorithm to assess for binding affinity to six relatively common class I HLAs that are distinct from each other (A*0101, A*0201, A*0301, A*2402, B*0702, B*4403) and eight Class II HLAs (DRB1*0101, DRB1*0301, DRB1*0401, DRB1*0701, DRB1*1101, DRB1*1301, DRB1*1501) with the expectation that resulting hits will provide coverage of a very broad

segment (98%+) of the human population. Class I epitopes were sorted by protein, allele, and EpiMatrix score to produce a short list (up to 25 for each category) of high-quality candidates.

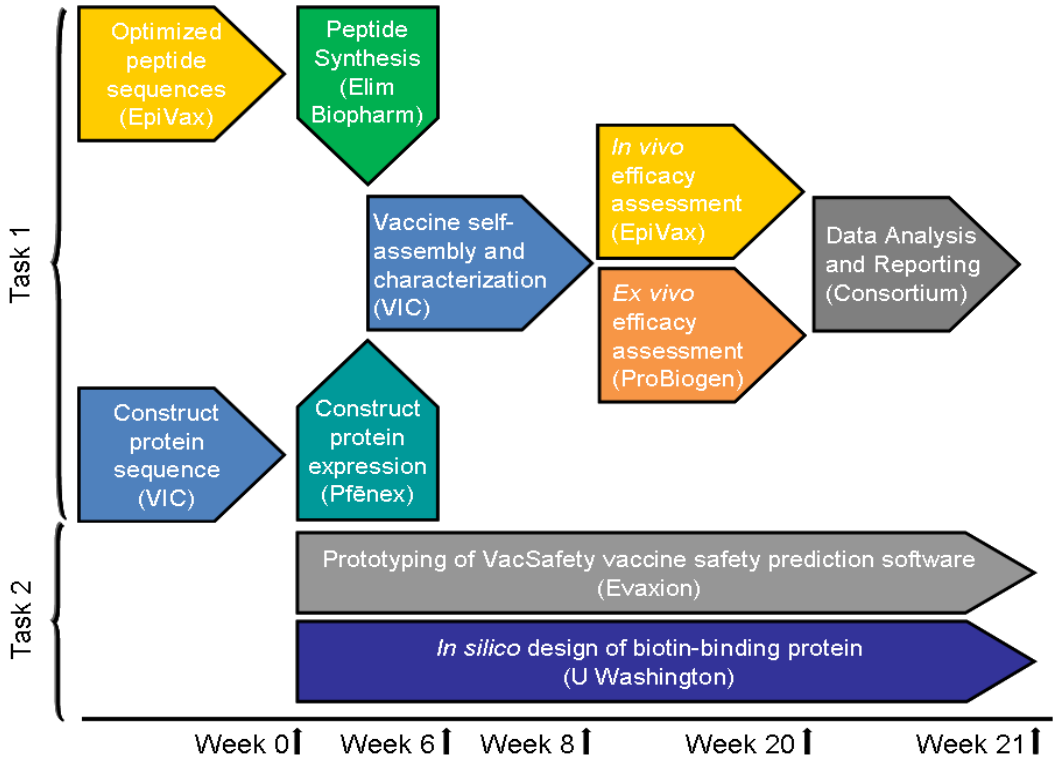


Figure 1: Timeline and Integration of Project Tasks

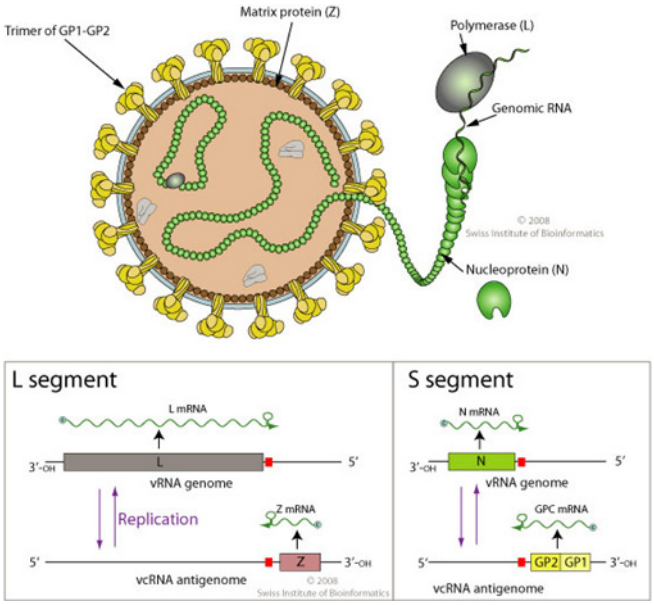
For Class II epitopes, EpiVax used their Clustimer algorithm to screen the results of the initial EpiMatrix analysis and identify putative T cell epitope clusters. These range from 15 to 25 amino acids in length and each may contain anywhere from 4 to 40 putative Class II-restricted binding motifs. One or more dominant T-cell epitope clusters can enable significant immune responses to even otherwise low-scoring proteins. Ranking of candidate clusters was done using a weighted scoring system. This analysis identified 91 such epitope clusters, which were further analyzed and adjusted, resulting in a final list of 107 candidate epitope clusters.

Step 2: Homology Analysis. EpiVax then compared each candidate epitope to a series of reference databases (including proprietary ones) and analyzed for homologies with human genome and commensal microbiotal genomes. Candidates with significant homologies were exluded from the candidate pool. Candidates were further screened against the IEDB database of known HLA ligands and T cell epitopes using a proprietary homology algorithm. The existence of homologues known to bind to HLA and/or to engage T cells helps to validate the immunogenicity predictions made by the EpiMatrix software. Candidate sequences that can be related to positive binding or T cell activation data are preferred in a vaccine. Finally, EpiVax constructed a database of known arenavirus isolates and used its homology toolset to look for sequences conserved across different arenaviruses.

Step 3: Candidate Finalization. Based on this process and the criteria described, EpiVax identified 24 Class I-restricted candidates (restricted by all six Class I alleles) and 12 Class II-restricted candidates. Candidates for antigen targeting within the Lassa virus include the G protein (with the precursor molecule glycoprotein C, GPC, post-translationally cleaved into a

stable signal protein, GS, and two glycoproteins GP1 and GP2), the nucleoprotein (NP), the L protein and the Z protein (Figure 2).

Figure 2: Proteins of the Lassa Virus



Source: Swiss Institute of Bioinformatics:
http://viralzone.expasy.org/all_by_species/212.html

We then excluded targets from the NP protein based on a reported potential for dysfunctional immune responses to the NP protein, which may potentiate viremia in Lassa (Fisher-Hoch *et al.* 2000; Qi *et al.* 2010; Hastie *et al*. 2011, 2012; Jiang *et al.* 2013). The candidates provided to the team for further analysis also included 12 class I and four class II targets within the L protein. After a review of the literature and a discussion with a Lassa virologist, our team ultimately decided not to include L protein targets based on the lack of any published evidence for its effectiveness as a target in Lassa. In their analysis, EpiVax also did not find any candidates from the Z protein that qualified for consideration. Our initial conclusion on the available published evidence was that a vaccine that included targets in both GP1 and GP2, but excluded targets in the NP, L and Z proteins, would still represent a protective vaccine.

2. Peptide Design and Synthesis

The key considerations in constructing the targeting peptides for the test vaccine were:

1. Limit class I and class II peptides to the Lassa glycoproteins.
2. Ensure peptides include class I and class II targets from both GP1 and GP2.
3. Ensure coverage of all six HLA superclasses with both the class I and class II peptides.
4. Maximize the number of peptides delivered per unit of HSP70.
5. Optimize the solubility of the peptide strings.
6. Combine class I and class II peptides in each peptide string.

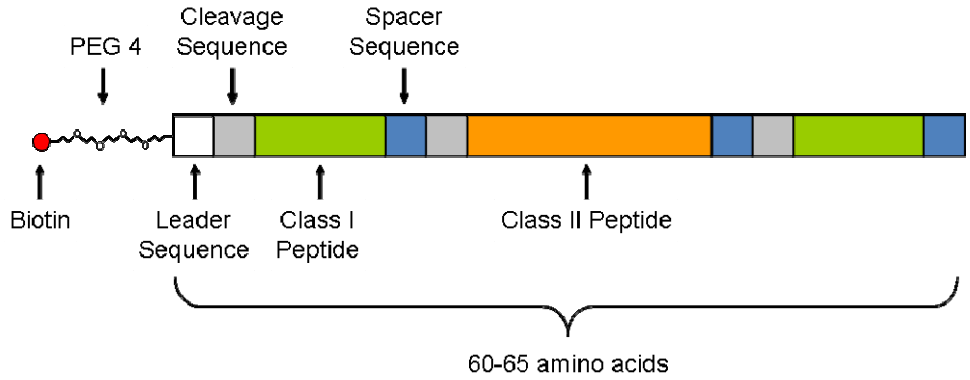
7. Include cleavage sequences to direct antigen processing and cross presentation.

8. Minimize junctional immunogenicity in the resultant peptide strings and creation of unintentional immunogenic epitopes.

Based on these design criteria, a final pepti de set—six long peptides of 60-65 AAs in length—was designed for the vaccine (Figure 3). The use of long peptides was based on a number of preliminary studies carried out before and in parallel with VaxCelerate II. These studies provided important evidence to support the use of this final long peptide structure. Figure 3A shows the template for each peptide, which concatenates two class I peptides to one class II peptide, with the class II peptides in the middle of the overall sequence. Spacer sequences are included to minimize junctional immunogenicity and improve solubility. Furin cleavage sequences are placed in between each class I and class II peptide to direct antigen processing in ways that enhance CMI responses in combination with the MtbHSP70-avidin construct. Figure 3C shows the Z-scores for each of the class I peptides selected across the six HLA superclasses. Six different class II peptides are utilized in this vaccine, with each peptide targeting one of the G proteins and each enabling high-affinity binding to MHCII proteins across all six HLA classes. The resultant vaccine is designed to induce both $CD4^+$ and $CD8^+$ T cell responses against targets on GP1 and GP2 across all six HLA superclasses, resulting in both short-term CMI responses and longer-term memory responses in vaccinated subjects.

Figure 3: Design of Peptide Sequences for Lassa Vaccine

3A: Template for Antigenic Peptides



3B: Peptide Sequences Selected for Vaccine

S1: LEQLE RVKR MLRLFDFNK AAY RVKR DHALMSI**IS**TFHLSIPNFN**Q**YE GPGPG RVKR LSDAHKKNLY AA -amide

S2: LEQLE RVKR GLYKQPGVPV AAY RVKR AQMSI**Q**LINKAVNALIND GPGPG RVKR FVFSTSFYL AA -amide

S3: LEQLE RVKR LINDQLIMK AAY RVKR GGKISVQYN**LS**HSYAGDAA RVKR **IT**EMLQKEY AA -amide

S4: LEQLE RVKR SYLN**ET**HFS AAY RVKR PIGYLGL**LS**QRTRDIY GPGPG RVKR TFHLSIPNF AA -amide

S5: LEQLE RVKR AA IPTHRHIV**G** AAY RVKR SFYL**IS**IFLHLVKIPTHRH GPGPG RVKR SPIGYLGLL AA -amide

S6: LEQLE RVKR SEGKDTPGGY AAY RVKR LFDFNKQAI**Q**RLKAEAQMS GPGPG RVKR AEAQMSIQL AA –amide

Red – Spacer sequences
Green – Class I and Class II peptide sequences
Blue – Pseudoprolines
Purple - Q(Dcmp), G(Dmb)

3C: Z Scores for Selected Class I Peptides

G Protein	AA Sequence	Peptide Length	Frame Start	Frame Stop	Z-Score A0101	A0201	A0301	A2402	B0702	B4403	Hits	Sum of Scores
GP1	LSDAHKKNLY	10	120	129	4.19	-1.31	2.05	-0.19	-0.29	-0.12	2	6.24
GP1	SPIGYLGLL	9	237	245	-0.04	1.36	-0.35	1.29	3.16	1.59	1	3.16
GP1	TFHLSIPNF	9	139	147	0.16	-0.89	0.55	3.38	1.13	1.92	2	5.29
GP2	ITEMLQKEY	9	411	419	4.59	-0.84	1.65	0.01	-0.11	-0.01	2	6.24
GP2	FVFSTSFYL	9	434	442	1.57	3.05	1.56	1.77	1.78	1.28	3	6.6
GP2	GLYKQPGVPV	10	478	487	0.17	2.23	0.51	-0.19	0.1	0.54	1	2.23
GP2	MLRLFDFNK	9	312	320	-0.24	0.99	3.22	0.4	-0.03	-0.62	1	3.22
GP2	LINDQLIMK	9	344	352	1.58	1.06	2.78	-0.39	-0.56	-0.97	1	2.78
GP2	SYLNETHFS	9	392	400	0.28	0.13	-0.07	2.48	-0.54	-0.57	1	2.48
GP2	IPTHRHIVG	9	452	460	-1.33	-1.28	-0.3	-0.33	2.44	-0.11	1	2.44
GP2	SEGKDTPGGY	10	269	278	1.84	-0.73	0.49	0.04	-0.12	3.51	2	5.35
GP2	AEAQMSIQL	9	328	336	0.18	0.53	-0.48	0.92	0.94	2.09	1	2.09

Once completed, the peptide design was provided to 21st Century Biochemicals for synthesis of the biotinylated peptides. They completed five of the six long (60-65 amino acid) biotinylated peptides, but the S2 peptide involved a difficult-to-synthesize section in the class II sequence. This synthesis problem resulted in an overall lower purity compared to the others. In the end, we substituted a shorter version of this peptide (leaving out the first class I peptide construct) in the final vaccine. The resulting vaccine therefore contained six peptides with a total of 6 class II and 11 class I peptides. 21st Century provided a certificate of analysis with each peptide that included results of mass spectrometry analysis (included as part of batch record). As part of quality control, MGH conducted characterization studies of these peptides for endotoxin levels, for their water solubility at 5 mg/ml, and for their ability to bind to streptavidin. The results of these studies showed that all peptides were soluble and bound to streptavidin.

3. Protein Expression

In parallel with the identification and synthesis of the peptides for the vaccine, Pfenex completed the expression and purification of the self-assembling protein construct (MtbHSP70-avidin). This process was similar to that applied in VaxCelerate I, but for this project the protein was completed under early-phase GMP standards agreed to by the parties and specified in the project-specific statement of work. A batch record for the production and a certificate of analysis for the final protein was provided with the product. As part of its quality control efforts, MGH conducted validation assays on the protein, including SDS-PAGE to gauge protein size and assess aggregation, assessment of endotoxin levels, ATPase activity and biotin-binding.

4. Vaccine Self-Assembly

The self-assembly and characterization of the final vaccine was completed in two days from the receipt of the final peptide set from 21st Century. We had improved both the upstream and downstream processes for self-assembly based on our experience with VaxCelerate I, and we implemented GMP and GLP processes for manufacture and characterization of the final vaccine. Self-assembly was completed quickly with no technical difficulties. Because of the increased solubility of the long peptides, self-assembly with MAV was completed rapidly and at high efficiency (greater than 98% self assembly within 60 minutes at room temperature using only water as the self-assembling solution). The final vaccine was formulated in PBS. Using near GLP assays, MGH verified that the vaccine met the standards for self-assembly, solubility, and activity. The vials of the test vehicles—PBS, self-assembling protein, peptide mixture and the self assembled Lassa vaccine—were shipped by express courier to MPI Research.

5. Mouse Vaccination Study

MPI prepared for the vaccination study by developing a protocol for the cGLP vaccination study in collaboration with MGH and EpiVax. In addition, MPI developed a validated GLP assay for measuring interferon gamma responses of splenic and lymphatic T cells to stimulation by flow cytometry. MPI initiated the animal study immediately upon receipt of the four different test vehicles from MGH. The study, based on an established timeline, took 6 weeks to complete. Initial data analysis from the study was completed within one week after the raw data from the mouse study was released by MPI.

The VaxCelerate team completed the "live fire" test in a timely manner, submitting the initial results to DARPA 123 days after receiving the Lassa fever genome. The project was completed within a three days of the 120-day goal in spite of a number of challenges including the need for a longer assessment of peptide choices based on unexpected L-protein hits, technical difficulties in production of one of the long biotinylated peptides, and the unexpected death of some of the mice due to a watering system issue. We believe that the team benefited from the conduct of the VaxCelerate I project that enhanced the coordination and communication among team members in the current project. The collaboration succeeded with the addition of two new member organizations, and the level of interactive problem solving was greater for this project.

This project demonstrated the ability, with proper team selection, planning and infrastructure, to integrate existing technologies together in order to implement a rapid vaccine design and testing process using a vaccine development platform that has potential for broad pathogenic application and scalability in the face of public health emergencies.

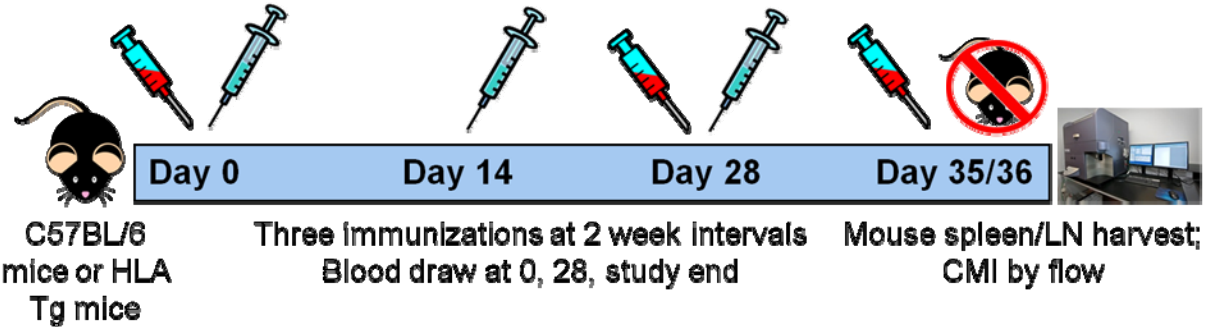
B. Significant Vaccine Immunogenicity

While rapid development of a vaccine is an important part of the VaxCelerate model, obviously development of a vaccine that shows efficacy is also key. Due to limitations in the scope of the project and time frame for execution, this project did not involve a challenge study. Rodents are carriers of Lassa and therefore not a proper animal model for challenge testing. In addition, challenge testing with Lassa requires BSL-4 containment. Therefore, this project focused on relevant immunogenicity responses. We used a transgenic HLA DR3 mouse model (mice with human HLA required as the peptides target human HLA) to assess T helper responses to pools of the Lassa class II peptides. The hypothesis was that the vaccine would induce significant interferon gamma expression by $CD4^+$ T cells in response to peptides.

The design of the mouse study followed a model developed by MGH and EpiVax during VaxCelerate I. This mouse vaccination model had shown positive results with a self-assembled influenza vaccine tested in parallel with the VaxCelerate II project. The mouse study compared three groups of 12 mice each: a saline control group, a peptide only group, a MAV protein group (self-assembled to a non-Lassa peptide), and the self assembled vaccine. Animals were vaccinated at 0 days and boosted at 14 and 28 days. Each mouse was vaccinated intradermally near the shoulder blade with 0.05 mL of vehicle on the right side and 0.05 mL of vehicle on the left side. Bloods were drawn at day -3 and day 28. Mice were sacrificed in two groups at day 35 and 36 of the study. Animal sacrifice and processing of the spleens and lymph nodes of the mice was conducted in two groups to ensure quality of throughput for the samples in light of the practical limitations of throughput at the MPI animal facility for this sample preparation. A final blood sample was taken and spleens and draining lymph nodes were removed. Splenocytes and lymphocytes were isolated and stimulated either with saline alone (negative control), PMA/ionomycin (positive control), or with a pool of the vaccine peptides (test condition). The pool included a mixture of only the six class II peptides in an acetylated, non-biotinylated form. Class I peptides were not included because the transgenic HLA mice used (DR3) do not have

the capacity to respond to human class I peptides. Flow cytometry was performed as described in the immunoassay protocol. A flow diagram of the animal study is shown in Figure 4.

Figure 4: Diagram of Mouse Vaccination Study

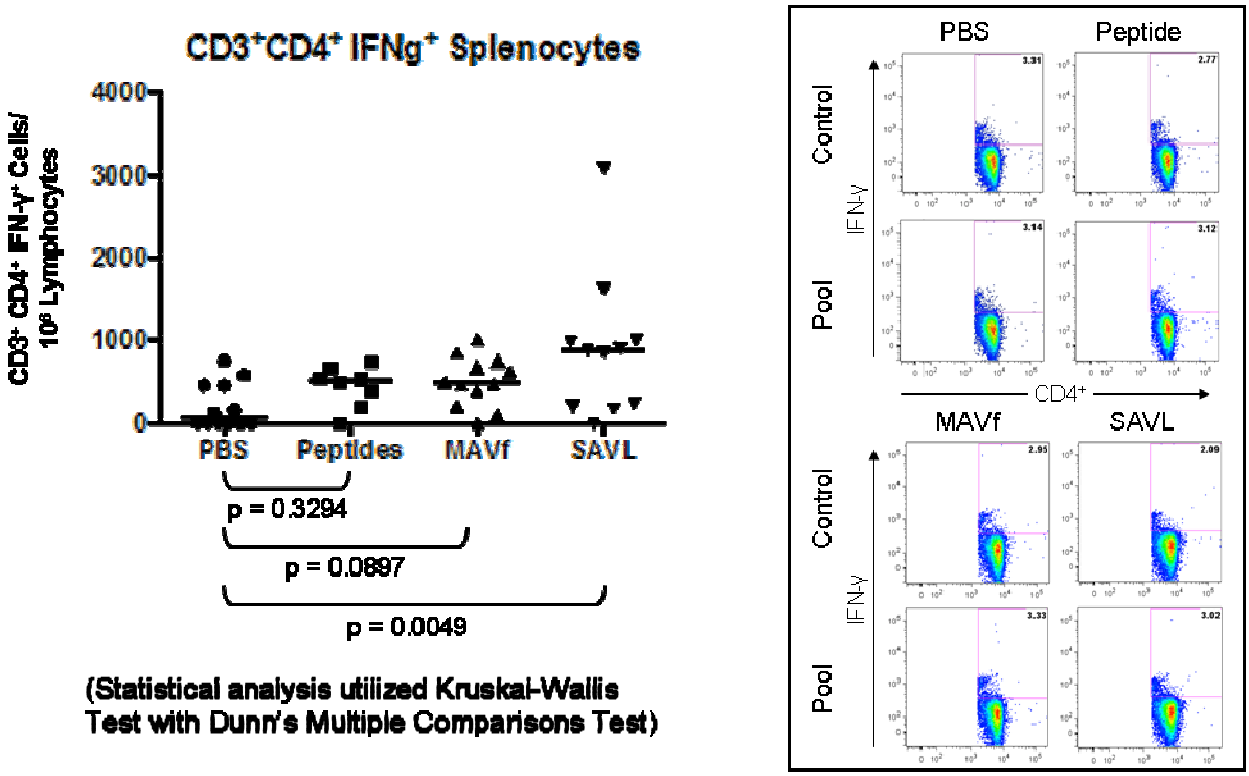


A total of 43 mice were processed for immunological end points: 12 from the saline (PBS)-injected mice, 8 from the peptide-injected mice, 12 from the self-assembled protein-injected mice, and 11 from the Lassa vaccine-injected mice. Following isolation and preparation of the splenocyte and lymphocyte samples from each mouse, the cells were stimulated with one of three reagents—medium alone (negative control), a prepared solution of all six class II Lassa peptides in amidated form (concentration of 10 µg/ml), or PMA/ionomycin (positive control)—for a period of six hours and then fixed and stained with fluorescent-labeled antibodies for extracellular markers $CD3^+$, $CD45^+$, $CD4^+$ and $CD8^+$, followed by permeabilization and staining with fluorescent-labeled antibodies for intracellular proteins interferon gamma (IFN-γ), IL-4, granzyme B (Gzm-B), and IL-17a.

Following completion of the immune cell isolation, stimulation and staining, cells were analyzed by flow cytometry on a Fortessa flow cytometer for the presence of different markers. Data was collected using FACSDiva software and analyzed in parallel by MGH (using FlowJo software) and by MPI Research (using FACSDiva). MPI utilized FACSDiva because FlowJo is not GLP validated for use with the Fortessa flow cytometry system. Cellular expression of interferon gamma (IFN-γ) was the cGLP-validated end point for the study. The other end points were not validated under the cGLP standards of the study. The process by which software analysis of flow results was to be performed was discussed by the team and harmonized between MGH and MPI. Resultant values for each mouse are expressed either as the number of net positive cells per 10^6 splenocytes or lymphocytes, or the percentage of net positive cells of the total $CD4^+$ or $CD8^+$ T cell population.

The results of the study were positive with respect to the primary end point for the study, showing that mice vaccinated with the novel self assembled Lassa Fever virus vaccine generated a statistically significant increase in splenic $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells in response to stimulation with the Lassa-specific class II peptides compared to mice exposed to PBS alone. The median values for the Lassa vaccinated mice were 891 $CD3^+CD4^+IFN\text{-}\gamma^+$ T cells per 10^6 splenocytes for the Lassa vaccine group (0.48% of the $CD4^+$ T cells). Splenocytes from mice vaccinated with either the peptide alone or with the MAV protein conjugated to a non-Lassa peptide also showed an increase in these values, but these were not statistically significant when compared to the control PBS exposed group. As will be discussed below, these comparative groups should not be considered negative controls. Statistical significance for each group compared to PBS vaccination was measured using the Kruskal-Wallis Test with Dunn's Multiple Comparisons Test, due to the inclusion of multiple test groups in the study. The summary of the $CD3^+CD4^+IFN\text{-}\gamma^+$ T cell data is shown in Figure 5.

Figure 5: Lassa Vaccine Induces a Significant Increase in $^{+}$IFN- γ^{+} from CD4+ T Cells in Response to Class II Lassa Peptide Stimulation



Other results from the study were not statistically significant, including $CD3^{+}CD4^{+}IL\text{-}4^{+}$, $CD3^{+}CD4^{+}Gzm\text{-}B^{+}$, and $CD3^{+}CD4^{+}IL\text{-}17^{+}$ responses in the spleen and all $CD8^{+}$ T cell responses. Since the vaccine peptides were targeted for human $CD4^{+}$ and $CD8^{+}$ T cell responses, and the mice were transgenic HLA DR3 only, no $CD8^{+}$ T cell responses were anticipated.

Analysis performed by MPI Research as part of its cGLP requirements yielded similar results, with a statistically significant population of $CD3^{+}CD4^{+}IFN\text{-}\gamma^{+}$ T cells in peptide-stimulated splenocytes from mice vaccinated with the Lassa fever vaccine. While the same approach to identifying cell populations from flow data were used, differences between the FlowJo and the FACSDiva software resulted in slightly different final cell counts. Data generated from the FACSDiva software will be included in the final cGLP study report issued by MPI (due in November) due to the validation requirements of their flow cytometry-based IFN-γ assay.

The immunogenicity end point for the study was selected for two reasons. First, the study required a measurement of cell-mediated immunity, specifically T cell responses. For Lassa fever, T cell responses and not antibody responses have been shown to be the key factor for viral clearance (Jahrling 1983; Auperin *et al*. 1988; Morrison et al. 1989; Fisher-Hoch *et al.* 2000; Botten *et al.* 2006). Second, effective $CD4^{+}$ T help is required for protective $CD8^{+}$ T cell responses. The transgenic HLA DR3 mouse model selected for this study enables us to look at Th1-oriented T help through expansion of the population of $CD4^{+}$ T cells that express interferon gamma in response to Lassa-specific peptides. The importance of $CD4^{+}$ T cells in response to arenavirus infection is supported by a number of animal studies done with lymphocytic choriomeningitis virus (LCMV). These studies have shown that virus-specific $CD4^{+}$ T cells play

an essential role in priming optimal $CD8^+$ T cell responses. Depletion or knock out of LCMV leads to the failure of LCMV-specific $CD8^+$ T cells to expand upon antigen re-encounter (Jannsen *et al.* 2003). In chronic LCMV infection models, $CD4^+$ T cell help is required to control viremia (Battegay *et al.* 1994; Matloubian *et al.* 1994). In addition to this T help function, some $CD4^+$ T cells have been shown to have direct effector function against LCMV infection (Jellison *et al.* 2005)

This experiment shows that the Lassa fever vaccine is capable of inducing a significant increase in interferon-gamma positive $CD4^+$ T cells in HLA transgenic DR3 mice compared to controls. The vaccine group showed strong positive values in splenocyte populations and not in lymphocyte populations. The $CD3^+CD4^+$IFN- γ^+ T cell responses were comparable to those reported in a number of other antiviral vaccine studies in mice, non-human primates and people, including two vaccines that achieved market approval in the U.S. Table 2 shows examples of these type of results for vaccines.

A more interesting comparison is to a study published on the identification of $CD4^+$ T cell epitopes against Lassa and other arenaviruses. In this study, Kotturi *et al.* (2010) injected HLADRB1*0101 transgenic mice intraperitoneally with with 10^7 PFU of recombinant vaccinia virus expressing different class II T cell epitopes for Lassa and a number of other arenaviruses. Seven days after inoculation, animals received a boost vaccination with the specific peptide. About two weeks later, the animals were sacrificed and splenic cells were examined for interferon gamma expression by CD4+ T cells using ELISpot. In these studies, the average percentage of positive cells across seven different arenaviruses to immunogenic peptides ranged from .03 to .39%. For Lassa, the average was .05%. The median response in the current Lassa study was higher than those seen in prior published studies.

Table 2: Comparison of Lassa Fever Vaccine $CD4^{+l}$FN- γ^+ T cell Responses to Other Vaccine Studies

Pathogen	Vaccine Type	Type of Subject	Immuno-assay	Response	Citation
Lassa	Protein-peptide complex	Tg HLA DR3 mice	Flow cytometry	891 cells per 10^6 splenocytes; 0.59% of total CD4+ T cells	Current study
Dengue	Attenuated virus	Humans	Flow cytometry	Up to 0.16% of CD4+ T cells	Lindow *et al. PLoS Neglec Trop Dis* 2012;6(7): e1742
Hepatitis C	Adjuvanted proteins	BALB/c mice	ELISpot	125-200 cells per 10^6 splenocytes	Sugauchi *et al. J Infect Dis* 2006;193(4):563-572.
HIV	BCG expressing Gag	Chacma baboons	ELISpot	0.4% of CD4+ T cells	Chege *et al. J Virol* 2013;87(9):5151
Smallpox	Attenuated live virus	BALB/c mice	Flow cytometry	0.1-0.3% of CD4+ T cells	Meseda *et al. Clin Vaccine Immunol* 2009; 16(9):1261
Yellow fever	Attenuated live virus	Humans	ELISpot	Up to 250 cells per 10^6 PBMC	James *et al. J Virol* 2013 epublished 09/18/13

It is worth noting that, while not reaching statistical significance, the peptide vaccinated group (without any adjuvant) showed an increase in response to stimulation with class II peptides. Previous studies we have conducted using shorter peptides in an influenza model (< 55 amino acids) did not show such responses. It is possible that the use of immunogenic peptides greater than 60 amino acids and featuring the use of furin cleavage sites may have the ability to promote immune responses by themselves. There are a small number of studies showing the vaccine potential of peptides of this length without the use of adjuvants (e.g. La Rosa *et al.* 2002). From this perspective, the peptide-only vaccination group should not be considered a negative control.

In addition, vaccination of mice with the MAV construct (self-assembled to a non-Lassa peptide) showed an increase in response to stimulation with class II peptides as compared to the PBS control. Again, this increase was not statistically significant. This response was somewhat similar to the potentiation of immune response seen in the HLA DR3 transgenic mouse influenza vaccine study we performed earlier. Previous experiments utilizing the self-assembled construct alone (MtbHSP70-avidin) have shown its immunopotency. In the VaxCelerate I project, it showed ability to non-specifically activate splenocytes from an HLA DR3 mouse in an ELISpot assay. It also showed an ability to stimulate human immune cells in the ProBioGen *ex vivo* artificial lymph node. The cytokine-stimulating effects of the molecule were distinct from those of the full self-assembled vaccine in that model. The self-assembling protein did not show such potentiating effects in our pilot studies on ovalbumin. These however, were performed in wild-type C57BL/6 mice.

We note that Haug *et al.* showed that HSP70 has the capacity to bind human HLA-DR molecules and induce proliferation of $CD4^+$ T cells (Haug *et al.* 2005, 2007; Fischer *et al.* 2010). This investigative team did not explore the implications of this activating HLA binding in the context of vaccines. We believe that the potentiation of the $CD4^+$ vaccine responses by the self-assembling protein in the context of this humanized HLA DR3 mouse model may be reflective of this activating binding capability of MtbHSP70 and may be one of the mechanisms of its vaccine potentiation in humans. This immunostimulating effect of MtbHSP70 seen only in transgenic HLA DR mice or in human samples also provides a concrete example of the limitation of the wild-type mouse models for predicting human immune responses and further emphasizes the value of developing more human-like preclinical models for testing vaccines.

Finally, we believe the accomplishment of this study is more impressive for being conducted within a cGLP environment by a contract research organization working with a validated immunoassay. The conduct of the study and performance of the assay was done by a third party outside the control of the participating vaccine platform development organizations and speaks to the potential for such results to be reproducible in other laboratories adopting this vaccination protocol. We think this result emphasizes the value of implementing quality control processes at the stage of preclinical research.

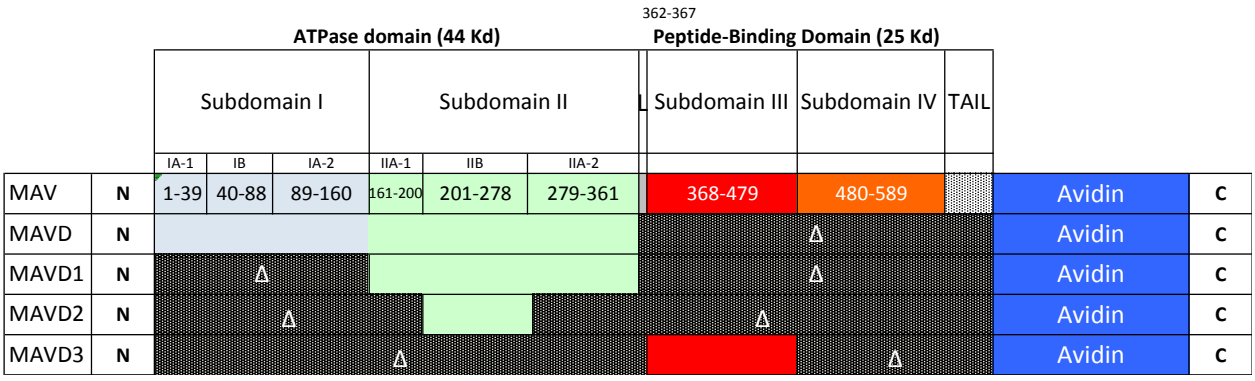
The overall approach of the VaxCelerate program was to use human-relevant end points for the assessment of vaccine efficacy and safety. Therefore, in keeping with this, an HLA DR3 transgenic mouse model was utilized. While the vaccine study marks a promising study end point for this Lassa vaccine candidate, this result itself is not a demonstration of protective efficacy. Rodents are not an appropriate model for assessing protective immunity in Lassa, since rodent species are carriers of the virus. Given adequate time and resources, there are additional evaluations that could be performed to better assess human responses to such a vaccine.

C. Characterization of MAV subunit proteins

The final key task involved an initial exploration of whether smaller subunits of the MtbHSP70 protein would retain the ability to cross-present antigens and stimulate antigen presenting cells as the whole protein does. HSP70 is composed of two major subdomains that are readily separable due to the fact they are joined by a flexible linker sequence. Each subdomain has additional subunits that may be isolated in a relatively intact fashion. The specific loci of a number of immunologic functions of MtbHSP70 have been identified, and the protein is known to contain sequences that induce immune tolerance. In this task, we assessed whether creating self-assembling proteins using a subunit of the full protein would retain the function of the full protein while eliminating potentially unwanted immunologic effects. We therefore isolated and characterized the morphology and immunologic function of a subunit-based self-assembling construct using a series of *in vitro* assays.

We first determined what subunit would be used for the initial test. We worked with the University of Washington team to analyze to what degree subunits of the HSP70 could be isolated while preserving their morphology. The first task was to enable initial testing of a smaller protein based on HSP70 that still preserved the ability of the protein to cross-present antigen. In MtbHSP70, this function has been reported to be localized within the ATPase domain (e.g., Huang *et al*. 2000; MacAry *et al.* 2004). We ended up selecting the whole ATPase domain of Mtb HSP70 (MAVD") and compared this to the full self-assembled protein ("MAV"). The plan was, based on success of this evaluation, to begin assessment of further subunits (Figure 6). Because MAVD did not pass initial assessments of immunologic activity, further subunit assessments were not pursued under this project.

Figure 6: Linear Structure of the Mtb HSP70 Variants

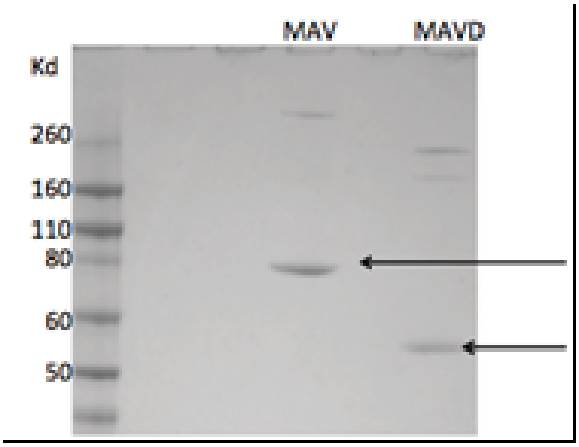


The MAVD-avidin protein was produced by the MGH Recombinant Protein core. The protein was produced using a *S. cereviciae* expression system. It was generated using a histidine tag to aid purification. The full MAV comparator had previously been expressed and purified by Pfēnex.

1. Molecular weight and purity

The resultant proteins were initially analyzed for mass and purity using SDS-PAGE. As shown in Figure 7, both proteins were of greater than 95% pure by gel electrophoresis standard. MAVD showed a band at its predicted molecular weight of 53.6 Kd (lower arrow). Both proteins were further characterized for their ability to hydrolyze ATP and bind biotin (see below).

Figure 7. Molecular Weight and Purity of MAV and MAVD on Gel Electrophoresis



400 ng of MAV or MAVD were mixed with sample loading buffer, heated to 70°C for 10 minutes prior to addition of dithiothreitol. The proteins were subjected to electrophoresis on a 4-12% Bis-Tris NuPAGE gel run in MOPS buffer at constant 200V for 50 minutes. Ten microliters of Novex® Sharp pre-stained protein standards used. Protein bands were identified by staining with RAPIDstain (Geno Technologies).

2. Biotin binding assay

We compared MAV and MAVD's ability to bind biotinylated-horseradish peroxidase (HRP) as a comparison of the functional normality of the avidin portion of the molecule. In this assay an excess of biotinylated-HRP was added to 6 nmoles of MAV or MAVD. After incubation for 15 minutes at room temperature, excess biotinylated-HRP was removed by addition of streptavidin magnetic beads. 100 μl of the supernatants were transferred to 96-well plate and 100 μl of tetramethylbenzidine was added. The plate was incubated in the dark for 10 minutes and the reaction was stopped by addition of 100 μl of 2N H_2SO_4. The results in Figure 8 show that MAVD had equivalent binding capacity by OD450 compared to MAV. This showed that the avidin in the MAVD was functional and matched the avidity of avidin in the original MAV construct.

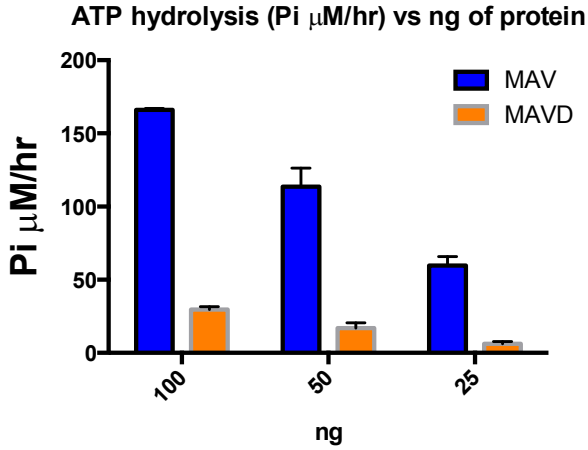
Figure 8: Comparative Biotin-Binding Capacity of MAV and MAVD

	OD450	
	no bHRP	**bHRP**
MAV	0.005	2.35
MAVD	0.006	2.519

3. ATPase activity

A test of the conformation of the MtbHSP70 is the use of an ATPase assay. HSP70 binds ATP at four binding sites within the ATPase domain, and the strength of the ATP-HSP70 complex is dependent on the conformation of the HSP70 molecule (e.g., Swain *et al.* 2007; Bhattacharya *et al*. 2009; Nicolaï *et al*. 2013). MAV and MAVD's ATPase activities were assessed using our ATPase assay. Three different concentrations of each protein were tested. The results are presented in Figure 9.

Figure 9: Comparative ATP Activity of MAV and MAVD



The ATPase assay was performed according to the manufacturer's recommendations (Innova Biosciences, ATPase Assay Kit cat# 601-0121). Briefly, 25 to 500 ng of assembled or unassembled MAV protein were incubated in 50 mM Tris pH 7.5 supplemented with 2.5 mM $MgCl_2$ and 0.5 mM ATP. The assay was carried out at 37°C for 20 minutes and stopped by addition of an acidic proprietary form of malachite green. Negative controls consisted of adding the protein mix after addition of the stop solution. Color was allowed to develop for 30 minutes at room temperature. Pi was measured based on the intensity of the OD at 650 nm.

MAVD demonstrated about 20% of the ATPase activity of MAV. This is consistent with published results relating to the ATPase domain. Removal of the C-terminal portion of the protein alters the overall conformation of the ATPase domain, resulting in a significant decrease in binding efficiency (e.g. Lopez-Buesa *et al.* 1998; Slepenkov *et al.* 2003).

4. DC maturation

MtbHSP70 is known to induce dendritic cell (DC) maturation, which contributes to its immunostimulative effect. We tested the ability of MAV and MAVD to stimulate DC maturation in a bone marrow-derived DC maturation assay. Bone marrow cells from C57BL/6 mice were expanded in RPMI containing 5% FBS supplemented with 10 ng/ml mGM-CSF and 1 ng/ml mIL-4 for 7 days. On day 7 CD11c positive cells were isolated using a magnetic beads loaded with CD11c specific antibodies. Cells were incubated with or without LPS (1 □g/ml), MAV (20 □g/ml) or MAVD (12.9 □g/ml) for 20 hours in a 37°C CO_2 incubator. At harvest cells were stained for CD11c and CD80 and analyzed by flow cytometry. Maturation was assessed based on the increase in CD80 expression on the surface of CD11c DCs. Results are shown in the figure 10. This result suggests that As can be seen from Figure 3, MAVD at 12 µg/ml failed to induce a maturation response in CD11c positive DCs. Twenty micrograms per ml of MAV had the same effect as micrograms per ml (the levels of LPS contamination in MAV was below 20 ng/ml). Deletion of subdomains III and IV affected the ability of MAVD to induce maturation of CD11c+ DCs.

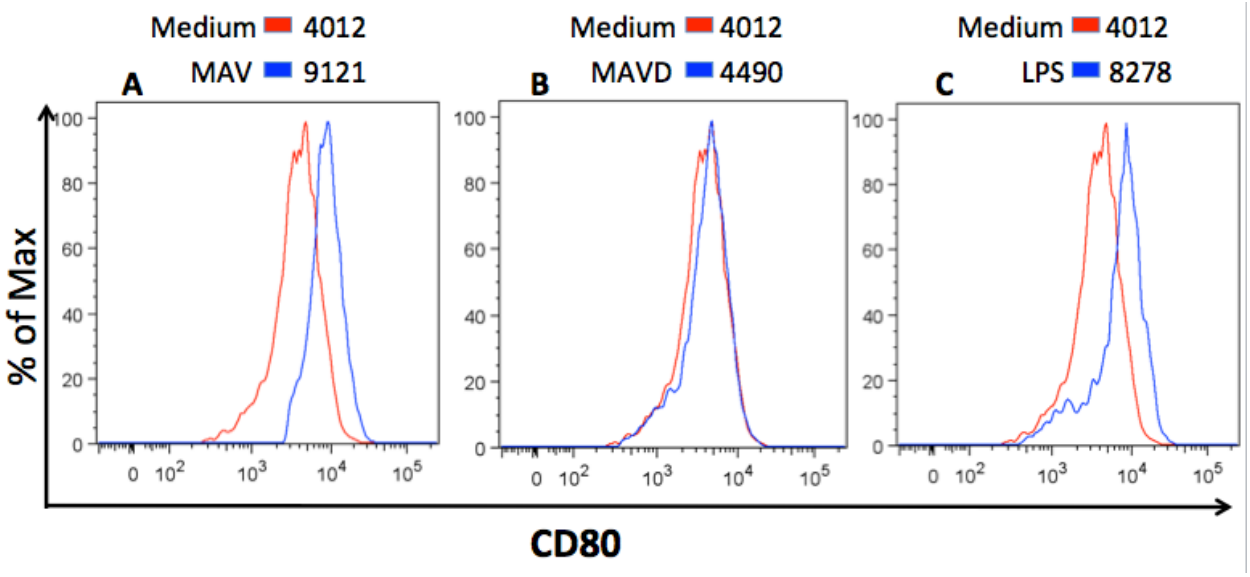
5. Cross-presentation

MtbHSP70 is known to induce antigen cross-presentation. This means that antigen entering DCs bound to MtbHSP70 are displayed on class I MHC. We designed an assay to assess the ability of MtbHSP70-avidin variants to support antigen cross-presentation. We exploited the availability of a fluorescently labeled antibody that recognizes SIINFEKL when it is bound to class I MHC of C57BL/6 mice (H-$2K^b$; BioLegend cat#141603).

We tested the ability of MtbHSP70-avidin to induce cross-presentation by using 2 peptides assemblies. In the first construct (SOVA) we used peptide: Biotin-PEG(4)-LEQLErvkrSIINFEKLrvkrISQAVHAAHAEINEAGR which contains both class I and class II ovalbumin specific peptides. The second construct consists of the SAV1 vaccine assembly. Since SAV1 does not contain SIINFEKL, staining with the PE anti-$H2K^b$ antibody should be negative. This is illustrated in Figure 11 where we see that cells exposed to SAV1 and medium

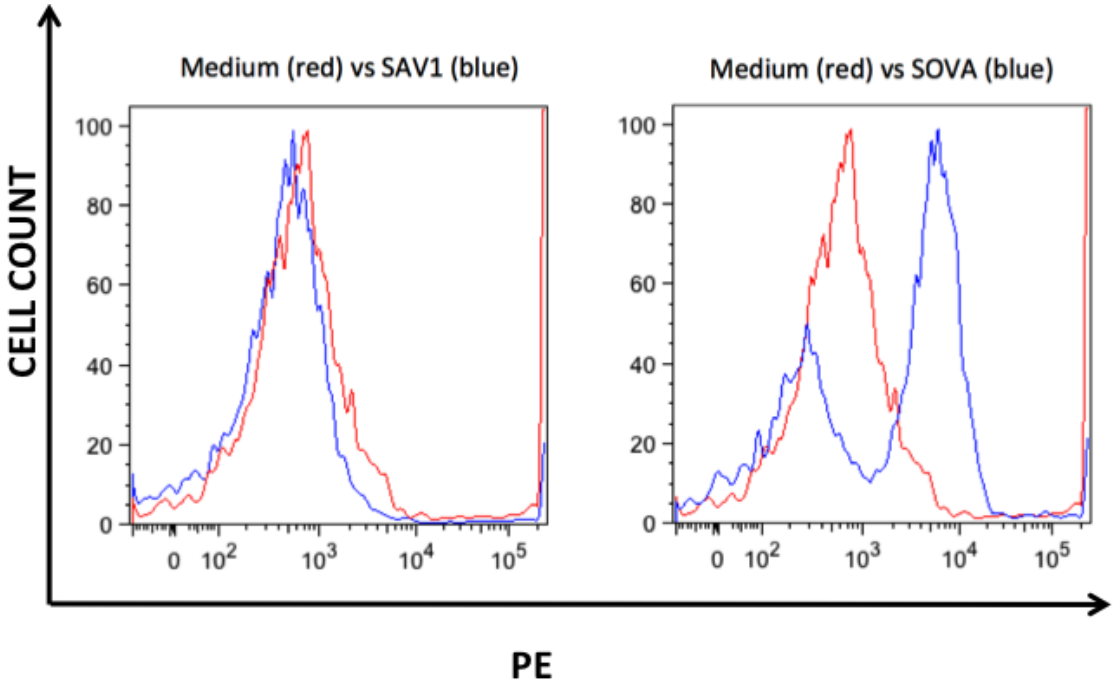
show the same fluorescence patterns while cells exposed to SOVA show a dramatic shift caused by the presence of SIINFEKL in the class I cleft.

Figure 10: DC Maturation Effect of MAV, MAVD and LPS



DC Maturation by MAV and MAVD. CD11c+ cells were incubated overnight with MAV (A), MAVD (B) or LPS (C). After harvested cells were stained for CD11c and CD80, fixed and analyzed by flow cytometry. Median fluorescence intensity (MFI) is indicated at the top of each graph.

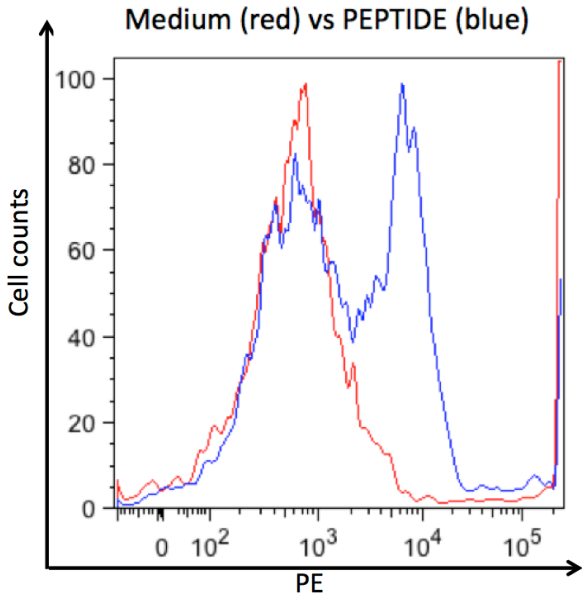
Figure 11: Assay Specificity for Detection of Cross Presentation



SOVA but not SAV1 induceS cross presentation in CD11c^{+} DC cells. CD11c+ DCs were prepared as described above. Cells were incubated for 12 hours with 10 μg/ml of SAV1 or 10 μg/ml of SOVA. After 12hours cells were harvested, stained and fixed prior to flow cytometry analysis.

However, it is important to note that our self-assembled constructs are incubated with a 10-fold molar excess of peptides. Since it is possible for external peptides to bind to class I MHC we needed assess the contribution of free peptide to the assay. This is illustrated in Figure 12 where it is evident that when Biotin-PEG(4)-LEQLErvkrSIINFEKLrvkrISQAVHAAHAEINEAGR is incubated with $CD11c^+$ DCs, there is an appreciable MFI shift.

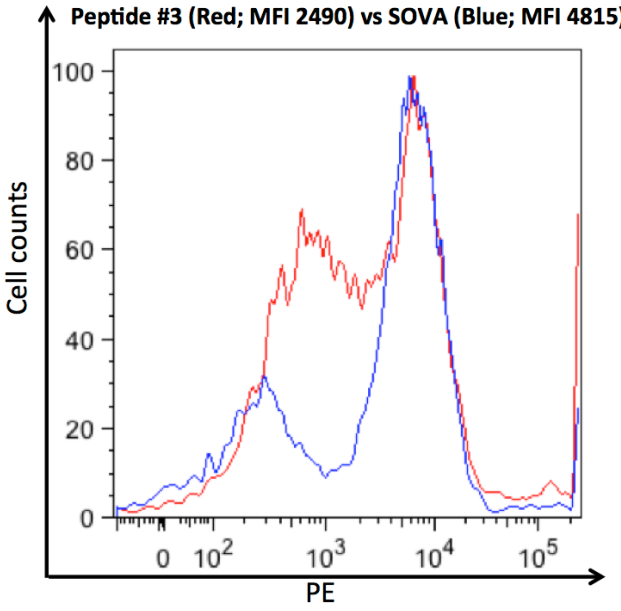
Figure 12: Contribution of Free Peptide to Cross Presentation in Assay



SIINFEKL containing peptides do not need to be processed to be presented on class I of CD11c+ DCs. Cells were prepared as described above. Cells were incubated for 12 hours with 2.5 µg/ml of Biotin-PEG(4)-LEQLErvkrSIINFEKLrvkrISQAVHAAHAEINEAGR. After 12 hours cells were harvested, stained and fixed prior to flow cytometry analysis.

In spite of this background effect, we could clearly observe a difference in the MFI between $CD11c^+$ DCs incubated with peptide alone and those incubated with SOVA (Figure 13). Incubation with peptide yielded an MFI of 2490 while incubation with SOVA resulted in an MFI of 4815.

Figure 13: Differential MHC Class I of Ova Peptides with and without HSP70

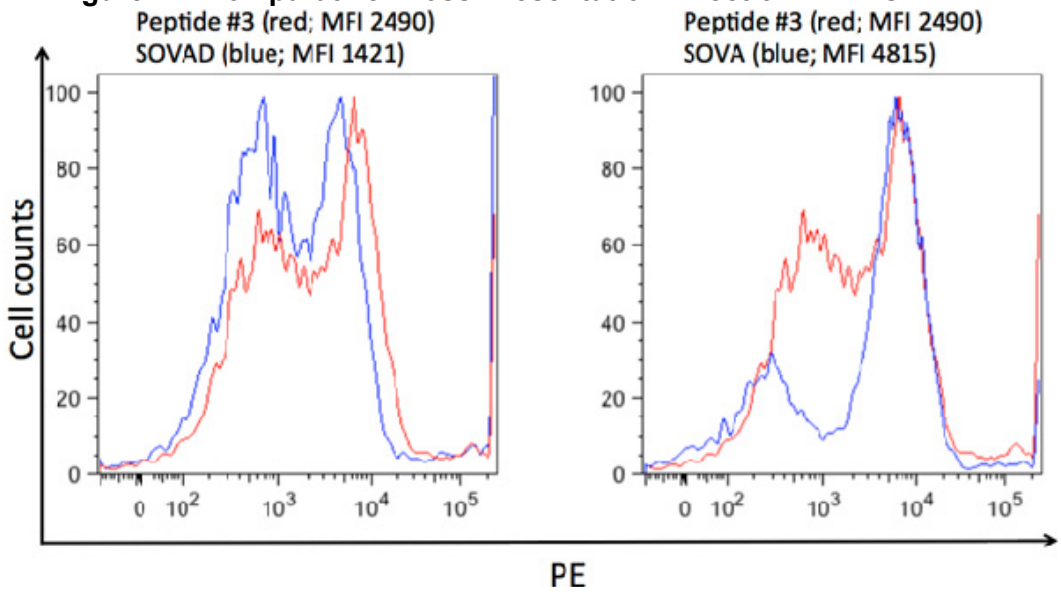


Differential detection of SIINFEKL on class I MHC of $CD11c^+$ DCs using Biotin-PEG(4)-LEQLErvkrSIINFEKLrvkrISQAVHAAHAEINEAGR and SOVA. CD11c+ DCs cells were incubated for 12 hours with 2.5 µg/ml of Biotin-PEG(4)-LEQLErvkrSIINFEKLrvkrISQAVHAAHAEINEAGR or 10 µg/ml of SOVA. After 12 hours cells were harvested, stained and fixed prior to flow cytometry analysis.

Comparing assembly of Biotin-PEG(4)-

LEQLErvkrSIINFEKLrvkrISQAVHAAHAEINEAGR with MAVD (SOVAD) or with MAV (SOVA) we observed that only SOVA induced cross-presentation in $CD11c^+$ DCs in this assay (Figure 14). This suggests that, in Mtb HSP70, the C terminal domain plays a significant role in DC maturation and cross presentation.

Figure 14: Comparative Cross-Presentation Effect of MAV vs. MAVD



MAVD does not stimulate cross-presentation in CD11c+ DCs. CD11c+ DCs were incubated for 12 hours with 10 μg/ml of SAVD or 10 μg/ml of SOVA. After 12hours cells were harvested, stained and fixed prior to flow cytometry analysis.

The MtbHSP70 ATPase domain-avidin fusion protein did not show an ability to cross present antigen in the *in vitro* assay and did not show great potential for immunostimulation through inducing DC maturation. This makes strategies that use the ATPase domain or subunits of this domain of questionable utility. We understand that some *in vivo* mouse studies have suggested that the ATPase domain can play a role in cross presentation but the evidence is equivocal (e.g., Huang *et al*. 2000; Udono *et al.* 2001; Liu *et al.* 2008; González-Vázquez *et al.* 2012). We recognize the limitations of an *in vitro* assay approach in defining such key mechanisms, but this approach provides a rationale strategy for de-risking potential new protein structures in terms of functional characteristics necessary to enhance vaccine responses.

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